Terpene emission in tissue culture

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Abstract

Tissue cultured plants' vessel headspace is subject to changes during subculture, the analysis of its variation offers a non-destructive approach for monitoring plant physiology. Among the volatile organic compounds (VOCs) that can be released by plants and be potentially recovered in the airspace of plant tissue cultures, terpenes have high importance and can offer a snapshot of the physiological status of the plant under *in vitro* cultivation. Terpenes are formed from carbon directly shunted from the photosynthetic carbon fixation cycle and are emitted under genetic and environmental control. The experiments described in this paper propose the evaluation of the plant terpene profile in the culture's headspace as an early indicator of plant stress and as a measure of the culture's physiological state through the characterization of plant terpene production. Monitoring of terpene emission as a plant response to mechanical stress such as plant wounding showed in the first hour after cutting an increased isoprene and monoterpene emission rates. The comparison of headspace composition of cultures of two fruit rootstocks, Colt and GF677, showed the first having higher emissions of isoprene, α -pinene and limonene as compared to the other one. During subculture a decreasing emission trend was observed, apparently as a result of culture aging. The *in vitro* headspace analysis of different myrtle (*Myrtus communis* L.) clones showed a specific and distinctive terpene emission profile. VOC monitoring of *in vitro* culture headspace is discussed as a non-destructive approach useful for evaluating the physiological activity of culture and for the determination of the potential production of terpenes.

Abbreviations: GC-MS – gas chromatography-mass spectrometry, VOCs – volatile organic compounds.

Introduction

While all plants exchange non-organic volatiles (CO_2, O_2) during photosynthesis or respiration, most of them also produce and emit in the atmosphere volatile organic compounds (VOCs), including alkanes, alkenes, ketones, aldehydes, alcohols, ethers, esters, carboxylic acids and terpenes (Kesselemeier and Staudt 1999; Tholl et al. 2006). Terpenes are the most abundant VOCs in unperturbed leaves (Guenther et al. 1995). The biogenic emission of terpenes has been extensively investigated *in vivo*, since these compounds are involved in the oxidative chemistry of the atmosphere (Andreae and Crutzen 1997) and for the important protective, defensive and info-chemical communication roles in planta, in plant-plant or in plant-pathogen/herbivore interactions (Langenheim 1994; Peñuelas et al.

1995; Paré and Tumlinson, 1997; Harrewijn et al. 2001; Peñuelas and Llusià 2004). Among the systems developed for VOC collection and monitoring headspace methods in combination with gas chromatography-mass spectrometry analysis have provided a more representative volatile profile of plants (Rapparini et al., 2001 and 2004a; Tholl et al. 2006). These methods can be used to study tissue culture headspace composition, that is a mirror of plant-environment interactions. Analyses of *in vitro* headspace focused on critical volatile compounds such as CO_2 and ethylene (De Proft et al. 1985; Buddendorf-Joosten and Woltering 1994) have provided basic knowledge regarding culture establishment, growth, aging and senescence. Other volatiles such as ethanol and acetaldehyde has been studied as related to photosynthesis and aging (Righetti et al. 1990). However, few studies have been conducted on volatile organic compounds, which would provide a more comprehensive knowledge of tissue culture dynamics. Investigations on headspace VOCs of *in vitro* cultures have been reported in olive (Williams et al. 1998), parsley (López et al. 1999), tomato (Maes et al. 2001; Maes and Debergh 2003) sweet orange (Alonzo et al. 2001), *Santolina canescens* (Casado et al. 2001; Casado et al. 2002) and cherry (Predieri et al. 1999).

VOC monitoring in the headspace of tissue culture vessel is a non-destructive analytical tool useful for the determination of the potential production of volatiles important e.g. for the pharmaceutical industry (Maes et al. 2001) and for evaluating the physiological activity of the culture, potentially providing information for a successful *in vitro* plant cultivation. Since the emission of terpenes by vegetation is driven by environmental factors of primary importance in micropropagation such as light and temperature (Sharkey and Loreto, 1993), the study of these compounds can provide details on tissue culture ecophysiology. Furthermore, terpene production is tightly connected to micropropagation technique, since it is induced by mechanical stress and injury such as stem wounding (Lewinsohn et al. 1994; Fall 2003). The potential of this information source for micropropagation was shown by Vercammen et al. (2001), who monitored emissions in culture headspace from *in vitro* mechanically wounded ivy and *in vitro* grown tomato plants under leafworm feeding, and studied differences in light and dark floral emissions of jasmine. Other applications of this approach have been developed by Maes and Debergh (2003) studying the emission of terpenes by tomato tissue cultures as a response to different stresses such as those induced by continuous light and by insect attacks.

In the present paper experiments are presented as related to the potential use of terpenes, recovered by *in vitro* headspace analysis, as early indicators of plant stress, as markers of the culture's physiological state and as examples of a non-destructive approach to the characterization of plant terpene production.

Material and methods
Headspace sampling and analysis
Glass jars (250 ml) commonly used for micropropagation ("Four seasons," Bormioli, Italy) were made suitable for headspace analysis by excluding any contact between the headspace and any reactive substances (e.g. rubber). An aluminium foil layer was placed on the jar's mouth before tightly closing it with a metal screw cap. Caps were equipped with a valve (Swagelock, Solon, OH, USA) to allow the introduction of a glass cartridge to break the aluminum foil and immediately enter jar headspace at sampling time. The other end of the cartridge was attached to a flowmeter, which in turn was connected to a vacuum pump. Gas samples of 250 ml were absorbed at a flow rate of 50 ml min^{-1} for 5 min from the culture jars onto Carbograph 1 (0.034 g) and Carbograph 2 (0.17 g) substrate (LARA, Rome, Italy) packed in a glass tube. Before sample collection, the traps were cleaned by passage of a stream of ultrapure helium at a flow rate of 300 ml min^{-1} and under heating up to 250°C. Sampling was done at room temperature under the hood. Cartridges were stored in a refrigerator at 4°C until analysis. Terpenes retained om carbon traps were then thermally desorbed and cryofocused at –150°C using a thermal desorption cold trap injection (Chrompack, Middleburg, The Netherlands). The desorption unit was connected to a 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) and using a 5970

quadrupole ion spectrometer (Hewlett Packard, Palo Alto, CA, USA) as detection system (Rapparini et al. 2004a). The desorbed sample, enriched in the cryofocusing unit, was transferred to the capillary column (60 m x 0.25 mm I.D; 0.25 μ m film of polymethylsiloxane; HP-1, Hewlett Packard) by heating the fused silica liner to 230 °C. The separation of terpenes was achieved maintaining the oven temperature at 40°C for 10 min and then increasing the temperature to 220°C at 5°C min^{-1}. Identifications of volatile compounds were based on the comparison of mass spectra and RIs of unknowns with those of authentic standards. Authentic standard compounds were supplied by Aldrich Chemical Co. (USA). Quantification of monoterpenes was performed using multilevel calibration curves, calculating the response factors for each compound and using d_{14}-cymene as internal standards (ISTD). The comparison of the response for the m/z 93 ion was generally used for quantification of monoterpenes, while 130 for the internal standard. Known aliquots of d_{14}-cymene were added to the sampling traps just before starting collection, allowing an overall normalization of the analytical system (sampling and MS response). Theconcentration of each compound is calculated after subtracting the average amount found in the control jars' headspace at the same sampling time and is expressed in relation to fresh weight of biomass at sampling time.

Medium composition

All media used contained MS macro- and micro-nutrients (Murashige and Skoog, 1962), LS (Linsmaier and Skoog, 1965) vitamins, sucrose 2% (w/v), agar (B&V, Parma, Italy) 0.65% (w/v). Colt and myrtle clones proliferation media was additioned with 1.46 µmol benzyladenine (BA) 0.49 µmol indole butyric acid (IBA). Myrtle rooting medium was additioned with 1.47 µmol indole butyric acid (IBA). Medium pH was adjusted to 5.7 with 0.1N KOH solution before autoclaving for 20 min at 120°C. Cultures were incubated in a growth chamber at 25±2°C with a 16-h photoperiod provided by cool white fluorescent tubes (Philips TLM 40W/33RS) at a photosynthetic photon flux of 68±2 µmol m^{-2} s^{-1}.

Plant material

Preliminary experiments were conducted on *in vitro* cultures of a number of cultivars and rootstocks of several temperate zone fruit trees: apple, pear, plum, peach and cherry. The culture that had the highest terpene emissions, cherry clonal rootstock Colt (*Prunus avium* X *P. pseudocerasus* Lind.) was used to study the effect of shoot cutting at transplant. For monitoring headspace variation during subculture, Colt was compared to a low-emitting culture, peach clonal rootstock GF 677 (*Prunus persica* X *P. amygdalus* L.). Terpene profiles were studied on three myrtle (*Myrtus communis* L.) clones different in origin and phenotype. For these experiments, 15 mm-long shoots were taken from 30-day-old cultures and placed, five per jar, in 250 ml "Four seasons" glass jars containing 50 ml medium. For each experiment three jars per treatment, arranged in a completely randomized design. were sampled. Experiments were repeated three times.

Immediate response

Colt shoots were taken from 30-days old proliferating cultures and two tranfer protocols were compared. One (AW) followed standard micropropagation techniques, microcuttings had callus and the basal part of the shoot removed with the scalpel before being transferred to a fresh medium, the other (NW) was directly transferred from the original jar to one containing the fresh medium without any wounding. After transplant, the jars were closed and headspace was sampled after 1, 3 and 24 h.

Subculture monitoring

In vitro cultures of the two fruit tree rootstocks were grown on a proliferation medium. Gas samples of 3 jars per cultivar per sampling date were taken on day 0, immediately after transplanting microcuttings, and at day 7, 14 and 21 of culture, in the middle of the light phase of the photoperiod (8-

hour). At each sampling time the headspace of three jars containing only culture medium, prepared as described for culture jars and kept in the same environmental conditions, were also sampled as a control of eventual headspace changes not induced by the presence of the explants.. Each experiment was repeated three times. After sampling, cultures were weighted and fresh weight determined.

Terpene profiles of different clones

Mirtle plant material was selected in two different Italian sites: two clones from Sardinia: C1, plants with small leaves and short internodes, and RUM 3, vigorous plants with large leaves and white berries; and one clone from Tuscany: C2, vigorous plants with standard-sized leaves and white berries. The headspace of these cultures was analyzed after 14 days of culture.

Statistical analysis

Data were analysed by ANOVA using PC SAS version 8.2 (SAS Institute Inc., Cary, NC, USA. Treatment were compared using the GLM Procedure with the LSD test. Differences referred to as significant had a P value less than 0.05.

Results and discussion

Immediate response

Headspace profile of Colt cultures mainly consisted of isoprene and different monoterpenes (Table 1). One hour after transplanting the Colt cultures, the total headspace terpene concentration was significantly higher in jars containing shoots subjected to scalpel wounding (AW treatment) as compared to non-wounded shoot culture (NW treatment). In particular, in the headspace of AW treated plants the isoprene and limonene concentration were 3 and 15-fold higher, respectively, as compared to the values found for NW treatment. Three hours after the transplant, no differences were found between the treatments, as was also found one day after transplant (Table 1) and during the following days of culture (data not shown). The headspace concentration of isoprene and monoterpenes observed after one hour appears as a direct response to wounding, since wounding causes the well-known burst of VOCs (Fall. et al. 1999; Holopainen, 2004; Loreto et al. 2006). Similar rapid responses of volatiles to wounding were observed by Maes et al. (2001) in *in vitro* culture of tomato plants. The increased production of terpenes upon wounding could have physiological functions, such as defence. In fact, stress conditions are counteracted by plants through an increase in radical scavenging processes (Grassmann et al. 2002) involving VOC emission (Loreto and Velikova 2001; Loreto et al. 2001).

Subculture monitoring

The analysis of the headspace of Colt and GF677 indicated the presence of isoprene, and the monoterpenes α -pinene and limonene, while camphene and traces of β -pinene, Δ -3-carene and p-cymene were present in Colt headspaces. In control jars, containing only culture medium, these volatile compounds were detected only as traces and with no variation observed during the experimental period (data not shown) .

In Colt culture headspace at day 0 only traces of terpenes were detected (data not shown), while at day 7 isoprene, α -pinene and camphene were present at concentrations close to 0.4 ng g^{-1} FW, while limonene was detected at lower concentrations (0.11 ng g^{-1}FW; Table 2). At day 14 isoprene, α -pinene and camphene concentrations were one half less than those recorded at day 7; while limonene was not detected. At day 21, the airspace concentrations of all the terpenes were lower than 0.1 ng g^{-1}FW (Table 2).

The headspace terpene concentrations of GF 677 cultures were lower compared to those detected for Colt cultures, and they were dominated by isoprene, that was the only terpene present at

day 7. At day 14 two monoterpenes were detected: α -pinene and limonene. At day 21 only isoprene was detected (0.1 ng g^{-1}FW).

Thus, from the observed results, the headspace terpene amount appeared dependent on plant species and culture aging. The observed different production of Colt and GF677 cultures are consistent with previous results obtained during *in vivo* studies of these two hybrid species, showing *Prunus avium* as a high emitter, while *Prunus persica* as a low emitter (Baraldi et al. 1998).

Isoprene and monoterpenes were the more abundant components in Colt headspace during the first period of culture, decreasing with culture aging. This observation appears to indicate the emission of terpenes as a signal of plant activity. Headspace of Colt tissue culture has been studied previously (Righetti et al. 1990; Righetti and Facini 1992) showing ethylene and acetaldehyde accumulation during subculture paralleling a decrease in CO_2 uptake. The observed results confirm these findings, since isoprene emission is positively correlated to photosynthetic activity and availability of nitrogen (Sharkey and Yeh 2001) the observed isoprene production decrease could indicate a reduced physiological activity as a result of leaf loss efficiency with aging and/or to reduced availability of nitrogen in the medium. Moreover, isoprene and monoterpenes emission is reported to decrease with leaf senescence (Guenther et al. 2000; Kuhn et al. 2004).

Terpene profiles found in the headspace of cultures of different myrtle clones

Isoprene and different monoterpenes, such α -pinene, camphene, limonene, were detected in the headspace of clones of *Myrtus communis* culture during the shoots' proliferating and rooting phases (Fig.1; Table 3). Isoprene and α -pinene were the dominant compounds in the culture headspace. However, each clone was characterized by its specific emission profile, both qualitatively and quantitatively, expressed as relative proportion (% of the total terpenes).

At shoots proliferating phase, clone C1 showed the highest percentage of isoprene (about 25%) and RUM3 the highest relative proportion of α -pinene (about 80%), as compared to clone C2. Similar differences were observed also during the rooting phase, even they were not statistically significant.

At both *in vitro* culture stages, the monoterpene 1,8-cineole was recovered in the headspace of clone C1 and RUM3, while it was not detected in that one of clone C2. However, clone C2 was characterized by the significantly higher percentage of Δ -3-carene and p-cymene, during both the proliferating and rooting phases. This clone showed the specific emission of γ -terpinene and camphor, compounds that were not detected in the headspace of the other two clones.

These results showed that each clone have a specific and distinctive terpene profile, which also confirms the analyses of *in vitro* and *in vivo* plant content as reported by Rapparini et al. (2004b). Studies of the analysis of essential oil composition of *Lavandula viridis*, show the presence of the same major components *in vitro* shoot-cultures and in the original field-grown parent plants, without remarkable compositional variations (Nogueira and Romano 2002), indicating that the analytical approach on *in vitro* cultures can provide useful information about the genetic potential of clones for the production of secondary compounds with significant value (Collin 2001).

Conclusions

Tissue culture headspace analysis has the potential to be a key non-destructive tool for monitoring plant physiological activity. In particular, terpene emissions can be studied for its correlation with culture physiological activity, e.g. terpene emission is directly linked to photosynthetic activity (Loreto et al. 1996; Peňuelas and Llusià 2002), and this could be of particular interest for monitoring tissue culture in semi-autotrophic and autotrophic conditions. Terpenes can be proposed as sensitive indicators of tissue-cultured plant quality together with the parameters proposed by Van Huylenbroeck and Debergh (2000) including photosynthesis, chlorophyll-a fluorescence, enzymatic activities, and carbohydrate pools.

Since terpene emission is affected by environmental factors, analysis of tissue culture headspace could represent a non-destructive method for studying environmental impact on cultures grown under standard growth conditions or under stress (e.g. tissue cutting at transplant, medium or atmosphere toxicity).

The analysis of *in vitro* headspace can also be used for the determination of VOC emission from different plant types with respect to the potential production of important volatiles e.g. for the pharmaceutical industry (Maes et al. 2001). This non-destructive approach can be useful when the preservation of the cultures is important, e.g. for mutant or somaclonal variant selection. New opportunities derive also from relatively recent methodology, e.g. the proton transfer-reaction-mass spectrometry (PTR-MS), which allows on-line real time analysis of the VOC emitted compounds at very low sensitivity (Lindinger et al. 1998; Warneke et al. 2003). Tissue culture technology would benefit from detailed views on the time courses of VOC profile development, which can provide a more comprehensive knowledge of headspace dynamics regarding plant culture metabolism, physiological status of cultures and plant-environment interactions.

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Table 1. Terpenes emission rate (ng g⁻¹ FW) from Colt *Prunus avium* X *P. pseudocerasus* directly transferred to a fresh medium (NW) of transferred after wounding (AW) after 1, 3 and 24 hours after transfer by *in vitro* headspace analysis. For each experiment three jars per treatment were used, experiment was repeated three times.

Time (h)	Transfer type	Isoprene	α-pinene	Camphene	Limonene	Total emission
1	NW	$0.280^b \pm 0.206$	$0.174^a \pm 0.084$	$0.139^a \pm 0.119$	$0.070^b \pm 0.037$	$0.487^b \pm 0.382$
	AW	$0.753^a \pm 0.056$	$0.570^a \pm 0.239$	$0.745^a \pm 0.341$	$1.090^a \pm 0.358$	$3.154^a \pm 0.895$
3	NW	$0.498^a \pm 0.014$	$0.667^a \pm 0.148$	$0.657^a \pm 0.165$	n.d.^{a}	$1.822^a \pm 0.322$
	AW	$0.340^a \pm 0.113$	$0.514^a \pm 0.194$	$0.570^a \pm 0.175$	n.d.^{a}	$1.424^a \pm 0.478$
24	NW	$0.678^a \pm 0.136$	$0.908^a \pm 0.288$	$0.795^a \pm 0.228$	n.d.^{a}	$2.381^a \pm 0.573$
	AW	$0.568^a \pm 0.218$	$0.696^a \pm 0.314$	$0.556^a \pm 0.225$	n.d.^{a}	$1.820^a \pm 0.711$

Values are means ± standard error. For each time, within each column means followed by different letters indicate significant differences by LSD test (P > 0.05). n.d. = not detected

Table 2. Total terpene emission rate (ng g⁻¹ FW) from Colt *Prunus avium* X *P. pseudocerasus* and GF677 *Prunus persica X P. amygdalus* after 7, 14 and 21 days of culture by *in vitro* headspace analysis . For each experiment three jars per treatment were used, experiment was repeated three times.

Root stock	Day of culture	Isoprene	α-pinene	Camphene	Limonene	
Colt						
	7	$0.449^a \pm 0.119$	$0.374^a \pm 0.124$	$0.372^a \pm 0.089$	$0.158^a \pm 0.092$	$1.353^a \pm 0.895$
	14	$0.231^{ab} \pm 0.059$	$0.189^{ab} \pm 0.091$	$0.225^{ab} \pm 0.071$	n.d.^{a}	$0.654^b \pm 0.322$
	21	$0.061^b \pm 0.042$	$0.011^b \pm 0.011$	$0.018^b \pm 0.011$	$0.013^a \pm 0.008$	$0.102^b \pm 0.478$
GF677						
	7	$0.131^a \pm 0.097$	n.d.^{a}	n.d.^{a}	n.d.^{a}	$0.131^a \pm 0.711$
	14	$0.199^a \pm 0.007$	$0.033^a \pm 0.012$	n.d.^{a}	$0.150^a \pm 0.074$	$0.382^a \pm 0.382$
	21	$0.108^a \pm 0.029$	n.d.^{a}	n.d.^{a}	n.d.^{a}	$0.108^a \pm 0.573$

Values are means ± standard error. For each rootstock, within each column means followed by different letters indicate significant differences by LSD test (P > 0.05). n.d. = not detected

Table 3. Percent composition of terpenes emitted by myrtle clones at 14 day of culture during shoot proliferating and rooting phase, by *in vitro* headspace analysis. For each clone culture medium, three jars per treatment were used, experiment was repeated three times.

Compounds	Proliferating			Rooting		
	C1	C2	RUM3	C1	C2	RUM3
Isoprene	20.6[a]± 2.3	23.5[ab]± 4.7	10.5[b]± 1.9	25.4[a]± 0.1	25.1[a]± 5.2	17.3[a]± 11.0
α -tujene	1.4[a]± 1.2	2.6[a]± 0.4	1.2[a]± 0.1	4.1[a]± 0.3	2.9[ab]± 1.0	1.2[b]± 0.5
α -pinene	69.5[b]± 2.5	67.6[b]± 3.7	81.4[a]± 1.3	62.9[a]± 2.0	61.2[a]± 3.2	75.6[a]± 10.3
camphene	1.1[a]± 0.7	0.6[a]± 0.3	n.d[c]	n.d[c]	1.2[a]± 0.5	0.9[ab]± 0.1
b-pinene	0.9[a]± 0.1	0.9[a]± 0.2	1.1[a]± 0.1	1.3[a]± 0.2	1.0[a]± 0.1	0.9[a]± 0.2
Δ 3-carene	0.3[b]± 0.2	1.1[a]± 0.2	0.4[b]± 0.0	0.5[ab]± 0.1	1.3[a]± 0.4	0.3[b]± 0.2
p-cymene	2.2[a]± 0.5	2.0[a]± 0.6	0.6[b]± 0.1	n.d[c]	4.3[a]± 2.6	1.5[b]± 0.1
1,8-cineole	2.1[b]± 0.4	n.d.[c]	4.0[a]± 1.0	3.7[a]± 0.6	n.d[c]	1.1[a]± 0.2
Limonene	2.0[a]± 0.6	1.1[a]± 0.4	0.7[a]± 0.4	0.9[a]± 0.1	1.9[a]± 0.9	1.1[b]± 0.2
γ - terpinene	n.d[a]	0.3[a]± 0.2	n.d[a]	n.d[b]	0.8[a]± 0.7	n.d[b]
camphor	n.d[a]	0.3[a]± 0.2	n.d[a]	n.d[b]	0.4[a]± 0.4	n.d[b]

Values are means ± standard error. For each stage of *in vitro* cultivation, within each row means followed by different letters indicate significant differences by LSD test (P > 0.05). n.d. = not detected

Figure Legends

Fig.1: Reconstructed mass chromatogram (on ions 93 and 130) of headspace of *Myrtus communis* clone C1 by preconcentration and gas chromatography-mass spectrometry analysis. 1: α -pinene; 2: camphene; 3: β -pinene; 4: β -mircene; 5: d_{14}-cimene (ISTD); 6: Δ -3-carene; 7: p-cimene; 8: 1,8-cineole; 9: limonene.

Fig. 2: Headspace sampling of *in vitro* culture.